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Counter-Intuitive Response to Water Limitation in a Southern European Provenance of *Frangula alnus* Mill. in a Common Garden Experiment

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Abstract: Climate change will intensify drought periods during the growing season in Western Europe. We mimicked this prediction by withholding water in summer from young rooted cuttings of *Frangula alnus* Mill., a common shrub species, originating from different latitudes in Europe (Italy, Belgium and Sweden) and growing in a common garden environment in Belgium. We followed the responses to the drought up to two years after the treatment. Counter-intuitively, the Italian provenance displayed earlier symptoms and stronger effects of water limitation than the other two provenances. A putative higher transpiration in this provenance could be suggested based on a relative larger shoot growth, larger leaves and a higher stomatal density. After the post-drought re-watering, the droughted plants of the Italian provenance entered leaf senescence later than the control plants, likely as a compensation mechanism for the lost growing time. Bud burst in the first year after the drought treatment and leaf senescence in the next autumn were both advanced in the drought treated group when compared with the control plants. Bud burst in the second year after the drought treatment did not display any differentiation anymore between control and drought treated plants. Growth traits also displayed legacies of the water limitation. For example, the drought treated plants showed a lower number of reshoots upon pruning in the year after the drought treatment. Our results suggest that assisted migration from southern Europe to western Europe as a climate change adaptation strategy might not always follow the expected patterns.

Keywords: drought; common garden; post-drought recovery; general linear mixed models; bud burst; leaf senescence; plant architecture; glossy buckthorn

1. Introduction

Chronic shortage of water can become life threatening to plants [1,2]. The increase in drought periods due to climate change, in terms of frequency, duration and severity, will impact the survival of plant populations and ecosystems in general, and thus the primary productivity and biodiversity worldwide [3–5]. Drought leads to a decreased water availability and hence to drought stress and a reduced fitness, augmenting the susceptibility to other biotic and abiotic disturbances [6–8]. A rising temperature increases the energy available for evapotranspiration and increases the water holding capacity of the atmosphere [9]. Climate change-induced warming therefore may result in more intense and persistent extreme climatic events like droughts [9]. This also implies that temperature is an important variable, next to precipitation, for understanding effects of global warming on vegetations and is included in the calculation of many drought indices [10].

Climate change-induced stressors such as drought and heat waves specifically menace the vitality of forest ecosystems [11–13]. Ultimately, the vulnerability to drought may lead to forest dieback [14,15] and biodiversity losses [16]. Understanding the responses of plants to drought stress can help to predict the changes in natural ecosystems that drought may cause. Because of the short time frame in which the recent climate change is occurring in comparison with past evolutionary changes [17], assessment of the magnitude of adaptive capacity in tree species is needed [18]. Although knowledge on interspecific variation of functional traits involved in tolerance towards heat and drought is important for predicting future species compositions of forests, these traits also vary within species [19,20]. To study the patterns of adaptive genetic variation within and among tree populations, trait variation studies in common gardens are needed [21–23]. A common garden experiment brings together populations from diverse geographic origins, allowing any population differentiation in traits to be attributed to genetic differences, as the populations are growing in the same environment. Broadly distributed forest tree species form genetically specialized populations, which are assumed to be (nearly) optimally adapted to a part of the climatic niche of the species [24].

Provenances growing at xeric sites have been suggested to be adapted to elevated temperatures and drier growth conditions than provenances from more humid sites. In a common garden experiment of *Quercus petraea* (Matt.) Liebl. saplings, the population from the more xeric home-site displayed least plasticity in morphological shoot, root and leaf traits in water limiting growth conditions, which was suggested to be an adaptation to drier growth conditions [25], following the hypothesis that populations growing in xeric environments are less plastic [26]. Populations from drier geographical origins grew better in a common garden with *Picea glauca* (Moench) Voss., displaying a higher resilience (ability to recover following a drought stress event) to extreme droughts as deduced from tree ring signals, compared with populations from more humid sites [27]. In *Pinus pinaster* Ait., a Mediterranean species, tree ring analysis showed that populations growing in more humid conditions displayed more growth resistance to drought, whereas populations growing at drier sites, although displaying a stronger growth reduction during water shortage, showed a faster post-drought recovery [28]. In *Fagus sylvatica* L., results from a common garden experiment suggest a better drought adaptation in marginal provenances [29]. Still, Arend, et al. [30] could not correlate climate at the home-site and response to drought in different provenances of three European oak species.

Drought stress reduces growth and affects the subsequent phenophases. Already in 1929 it was described that in Germany a summer drought of short duration may seriously reduce the height growth of *P. abies* (L.) Karst. and other tree species, and that repeated summer droughts may decrease stem growth for decades leading to enormous losses of increment [31]. Under stress conditions, carbon allocation gives lower priority to stem growth [32]. In this way woody plants redirect assimilates and energy to maintain respiration, to stimulate root growth over above-ground growth, or to favor other protective mechanisms, such as an increase in the non-structural reserve carbohydrates [33,34]. The pattern of growth reduction under drought stress can be species specific. Correlating tree ring widths with climate data in Switzerland revealed that radial growth was reduced in several dominant forest species (*Abies alba* Mill., *P. abies*, *F. sylvatica*) already in the year of water limitation itself,

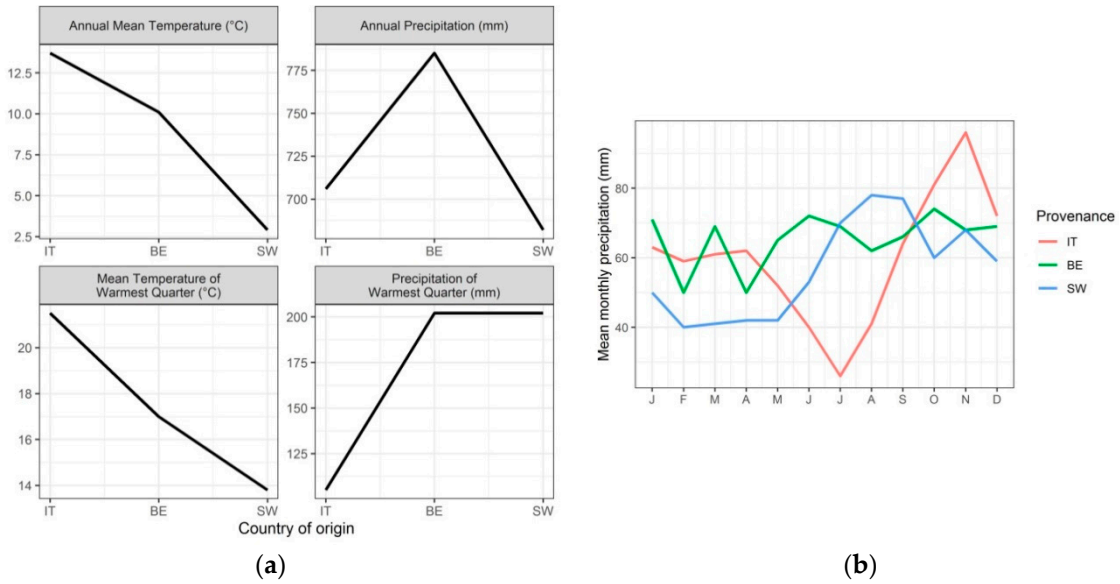
whereas for other species (*Quercus* spp.) the growth decreases were more clear in the years following the drought stress [35]. Trees may react on experienced drought by altering the timing of several phenophases. In general, height and radial growth stop earlier at the end of the growing season in drier environmental conditions [36–38]. A delayed autumnal senescence together with a delayed bud burst in the subsequent spring, were observed upon re-watering after drought stress in late summer in *Q. petraea* seedlings, which was suggested to compensate for the lost growing time during the drought period [39]. Analogously, in *F. sylvatica*, saplings exposed to summer drought showed upon re-watering a trend towards elevated starch concentrations and a stimulation of photosynthesis, likely compensating for the limitation of photosynthetic activity during the drought [40].

A significant annual average raise in temperature has already been observed in the northern part of Belgium and a further raise is predicted [41]. During the growing season, climate scenarios for this region predict a decrease in average precipitation together with an increase in extreme short rainfall events [41]. Our experimental set-up mimicked this prediction by withholding any watering to potted seedlings (heat waves) followed by plentiful re-watering (short heavy rainfall). Our study species is *Frangula alnus* Mill. (glossy buckthorn), a common, insect-pollinated and bird-dispersed shrub species (height up to 5 m) without economic value, and therefore little studied, but with large ecological benefits for the forest ecosystem, including a long flowering time, attracting many insect species. It is characterized by a wide distribution range throughout Europe, with genetic structures at different spatial scales as revealed by molecular studies [42,43]. As the local climate can vary significantly within the natural range of a tree species, it can be hypothesized that populations growing in a warmer climate may display a better drought-tolerance than populations from cooler sites, given a similar precipitation pattern. With a comparably lower precipitation during the growing season, this adaptation might be even stronger. We questioned whether different provenances of *F. alnus* expressed population differentiation in a water withholding experiment in a common garden environment. More specifically, we hypothesized that the provenance originating from the southerly located home-site may display a better adaptation to drought stress. For this we looked at the timing of visual stress symptoms, drought-induced growth reduction, post-drought recovery, and at the subsequent phenophases.

2. Materials and Methods

2.1. Source Material and Growth of Cuttings

Collection and germination of seeds from *F. alnus* was described before [44]. In short, seeds were collected in autumn 2011 from different mother plants in three provenances, following a latitudinal gradient in Europe: in Famelunga, Italy (lat.: 43.12181, lon.: 11.17654), in Heist-op-den-Berg, Belgium (lat.: 51.08424, lon.: 4.793124) and in Ottsjön, Sweden (lat.: 62.44210, lon.: 17.23451). The climate at the home-sites of the provenances is characterized by an annual mean temperature of 13.7 °C, 10.1 °C and 2.9 °C and an annual precipitation of 706 mm, 785 mm and 682 mm for the Italian, Belgian and Swedish provenances, respectively (data from WorldClim [45], Figure 1a). The maximum temperature of the warmest month was 28.4 °C, 22.3 °C and 19.9 °C and the minimum temperature of the coldest month was 2.7 °C, −0.3 °C and −13.3 °C for the Italian, Belgian and Swedish provenances, respectively [45]. A comparably lower precipitation during the growing season for the Italian provenance is displayed as a low value for the bioclimatic variable precipitation of the warmest quarter (150 mm, 202 mm and 202 mm for the Italian, Belgian and Swedish provenances, respectively; Figure 1a) and is also visualized in the mean monthly precipitation patterns (Figure 1b). Plants were grown in pots for two years in the nursery of the Research Institute of Nature and Forest in Geraardsbergen, Belgium, according to standard nursery techniques and using standard nursery materials. Plants were transplanted to a field trial in Gijzenzele, Belgium, in a single tree plot design (plants individually intermingled).



(**a**) (**b**)

Figure 1. Climate at the home-sites of the three provenances of *F. alnus* (data from Worldclim [45]). (**a**) Bioclimatic variables bio 1 (annual mean temperature), bio 10 (mean temperature of warmest quarter), bio 12 (annual precipitation) and bio 18 (precipitation of warmest quarter) are shown; (**b**) Mean monthly precipitation. IT: Italian provenance, BE: Belgian provenance, SW: Swedish provenance.

From the plants in the field trial (8 from Italy, 17 from Belgium and 14 from Sweden; each plant corresponds to a different genotype), eight cuttings per plant (clones) were taken in May 2016 and rooted in trays under transparent plastic in greenhouse conditions, following standard nursery techniques. For 1 Belgian and 1 Swedish genotype only 6 and 7 clonal plants were propagated successfully, respectively, resulting in a total of 309 plants. Each cutting was transferred to a 1 L pot (12 × 11 × 11 cm) using standard nursery potting soil (organic matter 20%, pH 5.0–6.5, Electrical Conductivity (EC): 450 µS/cm, dry matter 25%, fertilization: 1.5 kg/m^3 powdered compound fertilizer NPK 12 + 14 + 24) in the spring of 2017 and was further grown on a container field without additional fertilization. A total of 309 plants (64 from Italy, 134 from Belgium and 111 from Sweden) were individually intermingled on the container field. In May 2018, after leaf unfolding, all plants were pruned at 10 cm above soil level. This lower part of the plant below the pruning location is further called the base stem. The plants were a second time pruned one month later, leaving only one central shoot per plant. This allowed an easy handling and measuring of plants. After the drought experiment in the summer of 2018, the plants were further followed for growth and phenology (see below) till the spring of 2020. After the bud burst scorings in the spring of 2019, the plants were again pruned at 10 cm above soil level. Afterwards, all emerging shoots were allowed to grow (Figure 2).

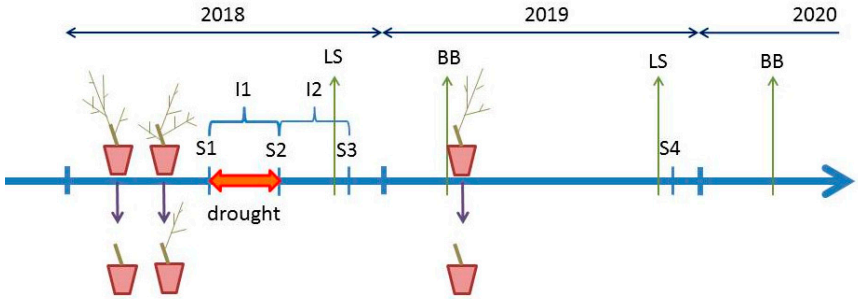


Figure 2. Time line indicating the drought period, pruning events and measurements on the plants during the years 2018 till 2020. S1 to S4: size measurements including length and diameter of the shoots. I1 and I2: increments of shoot length and diameter. BB: bud burst, LS: leaf senescence.

2.2. Water Withholding, Re-Watering, Wilting Score and New Leaves Score

For the water withholding treatment, all plants were transferred to the greenhouse. The pots were divided in two groups: a control and a treatment group, with each group containing four clones per genotype. All plants were individually mingled at random (completely randomized) in each group. The two groups of plants were soaked overnight from the 24th to the 25th of July 2018 (DOY 205 and 206) in two basins with the water level up to two cm above the bottom of the pots, reaching a fully water saturated condition. The potted plants were subsequently drained (an approximation of field capacity). From this point, any watering was withheld from the treatment group up to the 21st of August (DOY 233). During this period, the control plants were given water regularly. On the 21st of August, re-watering was performed by soaking all plants in two basins in the same way as at the beginning of the experiment. At the end of the drought period leaves had dried out for a significant amount of plants (38%). After the re-watering, all plants, including control and treated plants, were well watered. In the subsequent winter, all plants were transferred to the container field and both groups of plants were individually intermingled. They were kept there until the end of the study in the spring of 2020.

At the beginning of the experiment, after the drainage of the excess of water following the overnight soaking, all pots were weighted (DOY 206). Subsequently, pots were weighted weekly or be-weekly (in the treatment group on DOY 218, 225 and 233; in the control group on DOY 218 and 233) till the end of the treatment as an approximation for the water shortage experienced by the plants, the weight loss of the individual pots at the end of the drought period was calculated relative to the initial weight at the beginning of the treatment: relative weight loss = (weight DOY 206–weight DOY 233)/weight DOY 206 (Figure S1). This approximation is related to the soil relative water content (SRWC) which is the ratio between present soil moisture and field capacity [46], and is easily applicable to a larger amount of potted plants. The calculation did not take into account possible weight gain due to plant growth.

During the drought treatment, wilting and drying out of the leaves was observed on the 6th, the 10th, the 13th and the 21th of August 2018 (DOY 218, 222, 225 and 233) following a 5-level scoring protocol with 1: healthy plant, 2: leaves start to hang down, top of the plant still up right, 3: leaves hanging down, top of the plant also hanging down, 4: leaves start to dry out and green color is fading, 5: leaves are dried out.

After the re-watering, new shoots with new tiny leaves emerged on the stems of a certain amount of plants in the treatment group. The presence/absence of these new shoots was noted on the 4th of September 2018 (DOY 247). In addition, the location on the plant where these new shoots emerged was observed following a 4-level scoring protocol with 1: top of the central shoot (the upper third of the total length), 2: middle of the central shoot (the middle third of the total length), 3: base of the central shoot (the lower third of the total length), 4: base stem (below pruning location). When several new shoots emerged on different locations of the plant, the highest location was scored.

2.3. Size Measurements and Phenological Scores

The length and the diameter of the central shoot of all the plants were measured at the start of the experiment (DOY 206), after the re-watering (DOY 239) and in the succeeding winter 2018–2019 (Figure 2). As the plants were pruned up to one central shoot per plant, only the length of this shoot was measured, omitting the base stem of the plant. Diameter measurements were on the central shoot, 1 cm above the place where the shoot emerged from the base stem. Length and diameter increment of the central shoot during the treatment (from DOY 206 to DOY 239) and after the treatment (from DOY 239 till the growth stop in the winter of 2018–2019) were calculated by subtracting the length and diameter at the beginning of each period (DOY 206 and DOY 239) from the length and diameter at the end of each period respectively (DOY 239 and the winter stop). In the winter between 2019 and 2020, the length and the diameter (at 1 cm above the place where the shoot emerged from the base stem) of the largest shoot were measured again. The number of reshoots on the base stems were counted.

Two phenophases were scored two times on all plants: leaf senescence in the autumn following the drought treatment (2018) and in the subsequent autumn (2019), and bud burst in the springs of 2019 and of 2020. Leaf senescence was monitored following a 5-level scoring protocol that was specifically composed for this species: 1: 100% green leaves, 2: up to 33% yellow leaves, 3: between 33 and 66% of yellow leaves, 4: between 66 and 100% of yellow leaves, 5: leaves fallen. The scores were recorded on the 17th of October 2018 (DOY 290), on the top of the central shoot and on the base stem, leading to 2 scores per plant. In 2019 leaf senescence was scored according to the same scoring protocol, evaluating only the top of the plant, resulting in 1 score per plant. Scoring in this year was performed on three dates: on the 9th and 25th of September and on the 14th of October (DOY 252, 268 and 287).

The evolution of bud burst, leaf unfolding and shoot growth was recorded using a 6-level scoring protocol that was specifically composed for this species: 1: buds in winter rest, 2: swollen buds, 3: first green leaf emerging from the buds, but not yet unfolded, 4: up to 50% unfolded leaves; 5: 50%–100% of the leaves unfolded, 6: shoot growth. The scores were recorded on the 12th, the 17th and the 24th of April 2019 (DOY 102, 107 and 114). In 2019, plants were scored on two locations of the plant: on the apical bud (or, if the top of the plant had died off, on the highest emerging bud) and on the base stem, leading to 2 scores per plant. In addition to the bud burst score, the location where the first buds were flushing was scored on DOY 114 according to the same protocol used for scoring the location on the plant where the new shoots emerged after the drought treatment and the re-watering. In 2020, apical buds were scored (1 score for each plant using the same scoring protocol as the year before), on 3 days namely the 12th, the 17th and the 26th of April (DOY 133, 137 and 147).

2.4. Leaf Traits of the Three Provenances in the Control Group

The lamina length and lamina widest width were measured on the first fully developed leaf at the top of the central shoot of all the plants in the control group, shortly after the treatment, on the 3rd of September 2018 (DOY 246). On 8 plants (8 different genotypes) in the control group of each provenance, the density and the length of the stomata at the lower side of the first fully developed leaf at the top of the plant were counted and measured. Three transparent nail varnish prints were taken on the lower side of freshly collected leaves, omitting the leaf veins. The prints were put on a microscope slide and examined with a VANOX-S microscope (Olympus, Japan). With the free software Fiji [47], all stomata were counted within a given area [48,49]. Three such areas were chosen at random in one nail varnish print, resulting in 9 counts of the stomata for each leaf. In each of the 9 areas for each leaf, the lengths of 5 random stomata were measured with the Fiji program, resulting in 45 stomata length measurements for each leaf.

2.5. Statistical Analysis

The statistical analyses were performed in the open source software R 3.6.1 [50] using linear and general linear mixed models. As our main goal was to look at differential responses to drought stress among the three provenances, provenance (P, categorical variable with three levels) was in the fixed part of the models. The Belgian provenance was the standard to which the Italian and the Swedish provenance were compared. When relevant, the relative weight loss of the pots from the water withholding treatment (W, linear variable), as an approximation for the water limitation that the seedlings experienced, and the day of observation (D, linear variable) were added as co-variates in the models.

2.5.1. Linear Mixed Models

Shoot length and shoot diameter were modelled using linear mixed models in the package lme4 [51].

The length and diameter of the central shoot (S_1) at the start of the experiment were not yet influenced by the treatment. Therefore, these two size measurements were modelled with only provenance (P) in the fixed part of the model (2 models, each 309 observations):

$$S_1 = \alpha_{S1} + \beta_{PS1}P \quad (1)$$

The genotype and the clone nested within genotype were in the random part of the models. To examine the distribution of the modelled variance, models were fitted for the length and the diameter of the central shoot at the start of the experiment, having no explanatory variables in the fixed part, and having the provenance, the genotype nested within the provenance, and the clone nested within the genotype in the random part.

The growth increments of both the length and the diameter of the central shoot during the drought treatment (I_1), and after the treatment until the winter growth stop (I_2, Figure 2), were modelled (4 models, each 309 observations):

$$I_1 = \alpha_{I1} + \beta_{PI1}P + \beta_{WI1}W + \beta_{PWI1}PW + \beta_{S1I1}S_1 \quad (2)$$

$$I_2 = \alpha_{I2} + \beta_{PI2}P + \beta_{WI2}W + \beta_{PWI2}PW + \beta_{S2I2}S_2 \quad (3)$$

S_1 is the length or the diameter of the central shoot at the start of the treatment (increment of length or diameter, respectively) and S_2 at the end of the treatment. A significant interaction term between relative weight loss of the pots and the provenance indicated that the change in increment growth due to the drought treatment was different among the three provenances. The genotype and the clone nested within genotype were in the random part of the models.

The length and the diameter of the largest shoot that emerged on the base stem (S_4, Figure 2) were measured at the end of 2019 and were modelled (2 models, each 244 observations):

$$S_4 = \alpha_{S4} + \beta_{PS4}P + \beta_{WS4}W + \beta_{PWS4}PW \quad (4)$$

No initial height or diameter was added in the fixed part of the model as the plants were pruned at the beginning of the growing season. The genotype and the clone nested within genotype were in the random part of the models. To examine the distribution of the modelled variance, the same models were fitted, except that provenance was moved from the fixed to the random part of the models.

Leaf lamina length and lamina widest width (L) were modelled (2 models, each 155 observations):

$$L = \alpha_L + \beta_{PL}P + \beta_{H2L}H_2 \quad (5)$$

The genotype and the clone nested within genotype were in the random part of the model. H_2 is the length of the central shoot at the end of the drought treatment. To examine the distribution of the modelled variance, the same models were fitted, except that provenance was moved from the fixed to the random part.

Stomatal density and the length of the stomata (St) were modelled (2 models, 216 observations for stomatal density, 1080 observations for stomatal length):

$$St = \alpha_{St} + \beta_{PSt}P + \beta_{H2\,St}H_2 + \beta_{LaSt}L_a \quad (6)$$

L_a is the lamina length of the leaf. As only plants with unique genotypes were chosen for this analysis, the random part consisted of genotype, next to image nested within genotype (nail varnish prints on which stomatal traits were examined). To study the distribution of the modelled variance, the same models were fitted, except that provenance was moved from the fixed to the random part of the models.

2.5.2. General Linear Models

The presence/absence of new shoots with new leaves emerging after the post-drought re-watering was modelled with a general linear mixed model. Only plants from the treatment group were included as among the control plants this phenomenon was not observed. Plants that died during the drought treatment were also excluded from the model. The chance (p_1) for new shoots emerging after the post-drought re-watering was calculated as follows (112 observations):

$$\log(p_1/(1 - p_1)) = \alpha_{p1} + \beta_{Pp1}P + \beta_{Wp1}W + \beta_{PWp1}PW + \beta_{H2p1}H_2 \quad (7)$$

The genotype was in the random part of the model.

The wilting scores were ordinal and ordered chronologically from healthy plants (level 1) up to plants with only wilted and dried out leaves (level 5). Therefore, the scores were modelled with cumulative logistic regression in the package ordinal [52]. The function "clmm" in the package ordinal fits cumulative link mixed models to ordinal data. The cumulative probability of the ith observation having the jth level or below is modelled, where i represents all observations and j represents the response levels (wilting scores 1 to 5). Thus, the cumulative probability is the chance to maximally have reached a given level of the ordinal response variable. This chance (p_2) was calculated as follows (154 observations):

$$\log(p_2/(1 - p_2)) = \alpha_{Tp2} - \beta_{Dp2}D - \beta_{H1p2}H_1 - \beta_{Wp2}W - \beta_{H1Wp2}H_1W - \beta_{Pp2}P \quad (8)$$

with α_{Tp2} as an estimated threshold value for the passing on from one level of the ordinal wilting score to the next. A significant interaction term between length of the central shoot at the start of the treatment and the relative weight loss of the pots during the treatment indicated that the wilting response of the plants upon the drought treatment depended on their height. Genotype and clone nested within genotype were in the random part of the model.

The phenological scores were also modelled using cumulative logistic regression in the package ordinal [52]. For bud burst, the scores were ordered in a reversed way, from the end of the event till the beginning: from unfolded leaves to buds in winter rest (from 6 to 1) and for leaf senescence the scores were ordered in a normal chronological way, from green leaves to leaf shedding (from 1 to 5). The reversion of the order of the bud burst score levels allowed an easier interpretation of the modelled probabilities. A chance to have reached maximally score 4 in a bud burst variable that is ordered from 6 to 1 indicated a chance to have reached score 6, 5 or 4. This indicated that plants displaying early bud burst (higher score levels than other plants on a given day) have higher chances in the modeling than the others.

For leaf senescence in 2018, the chance (p_3) to have reached maximally a given phenological score level on the day of observation was calculated following a cumulative logistic regression (241 observations):

$$\log(p_3/(1 - p_3)) = \alpha_{Tp3} - \beta_{Pp3}P - \beta_{Wp3}W - \beta_{PWp3}PW - \beta_{H3p3}H_3 \quad (9)$$

H_3 is the length of the central shoot at the end of the growing season 2018. A significant interaction term between relative weight loss of the pots and the provenance indicated that the change in the timing of the leaf senescence due to the drought treatment was different among the three provenances. Genotype was in the random part of the models.

For leaf senescence in 2019, a similar model was applied, but with addition of the day of observation in the fixed part and replacing H_3 with the length of the largest shoot on a plant at the end of 2019 (H_4). Clone nested within genotype was added in the random part to account for the repeated measurements on the same plants (3 observation days, 734 observations).

Bud burst scores in both 2019 and 2020 were also modelled with cumulative logistic regression (713 observations):

$$\log(p_4/(1 - p_4)) = \alpha_{Tp4} - \beta_{Dp4}D - \beta_{Pp4}P - \beta_{Wp4}W - \beta_{PWp4}PW - \beta_{Hxp4}H_x \quad (10)$$

H_x is the length of the central shoot at the end of the growing season 2018 (bud burst scores in spring 2019, H_3) or is the length of the largest shoot on a plant at the end of the growing season 2019 (bud burst scores in spring 2020, H_4). Genotype was in the random part of the model, together with clone nested within genotype.

The number of shoots (N) that emerged after pruning the plants back to the base stem, which was conducted after the leaf unfolding in spring 2019, was modelled using a Poisson regression (245 observations):

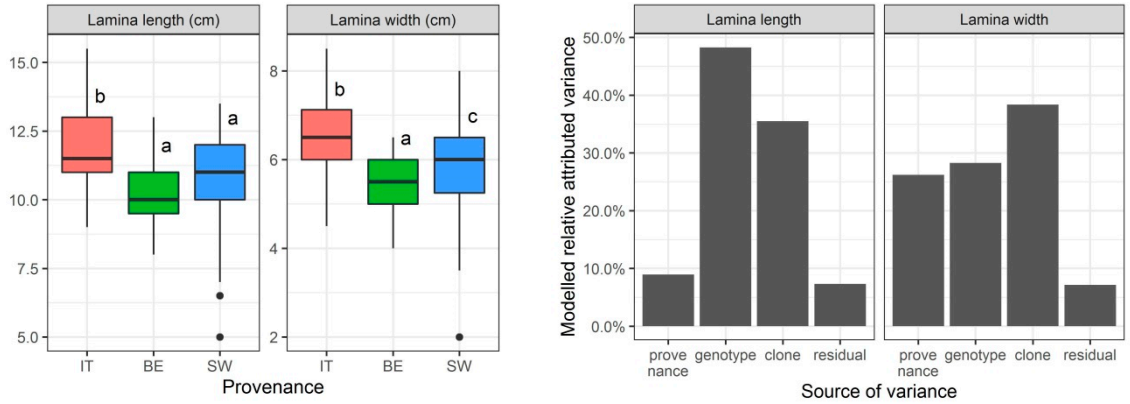
$$\log(N) = \alpha_N + \beta_{PN}P + \beta_{WN}W + \beta_{PWN}PW + \beta_{H4N}H_4 \quad (11)$$

The genotype and the clone nested within genotype were in the random part of the model.

3. Results

3.1. Leaf Lamina Size and Stomatal Traits

Leaf traits were examined among the control plants to characterize the three provenances. Leaves from the Italian provenance were significantly longer and wider than the Belgian provenance (p-value of 0.034 and <0.001 for P_I respectively in Table S1, Figure 3a). Leaves from the Swedish provenance were significantly wider than the Belgian provenance (p-value of 0.009 for P_S in Table S1) but not longer (p-value of 0.099 for P_S in Table S1). Modelling the relative distribution of variance for the length and the width of the leaves, the two factors provenances and genotypes within a provenance (genetically controlled) explained more variation than the factor clones within a genotype (Figure 3b).

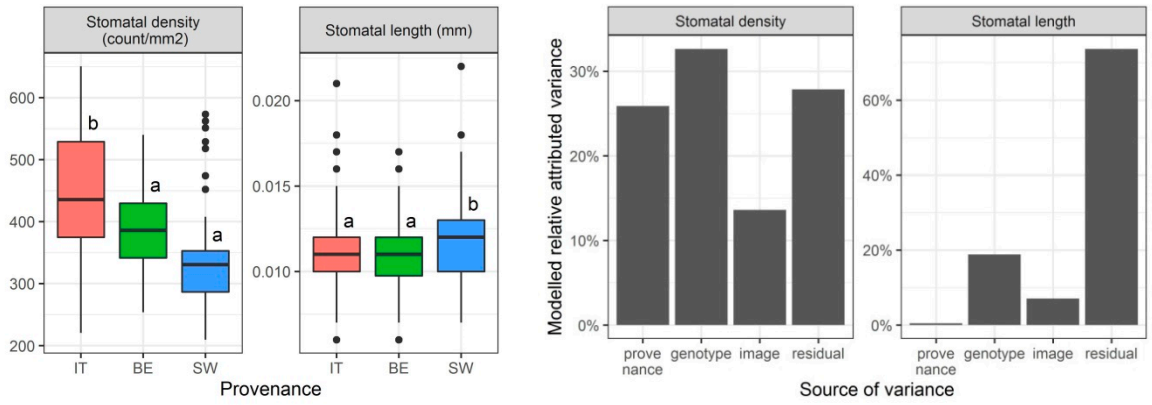


(**a**) (**b**)

Figure 3. Leaf lamina length, lamina widest width and their ratio for the three provenances. (**a**) Box plots of the measurements. Letters indicate significant differences (Table S1); (**b**) Modelled distribution of the relative variance. Variance is explained by the factors provenances, genotypes within a provenance and clones within a genotype. IT: Italian provenance, BE: Belgian provenance, SW: Swedish provenance.

Leaves of the Italian provenance had a significantly higher stomatal density than the Belgian provenance (p-value = 0.017 for P_I in Table S2), whereas stomatal density of the Swedish provenance did not differ significantly from the Belgian provenance (p-value = 0.273 in Table S2, Figure 4a). The length of the stomata of the Italian provenance did not significantly differ from the stomata length of the Belgian provenance (p-value = 0.283 for P_I in Table S2, Figure 4a). However, a trend was present for the leaves of the Swedish provenance to produce larger stomata than the Belgian provenance (p-value = 0.046 for P_S in Table S2). For the modelled variance of stomatal density, the contribution

of the factors provenances and genotypes within a provenance together (genetically controlled) was higher than the variance attributable to the three different nail varnish prints taken for each leaf to count the stomata (Figure 4b). Residual variance was however relatively large. For stomata length, the residual variance was extremely large, indicating little genetic control in this case (Figure 4b).

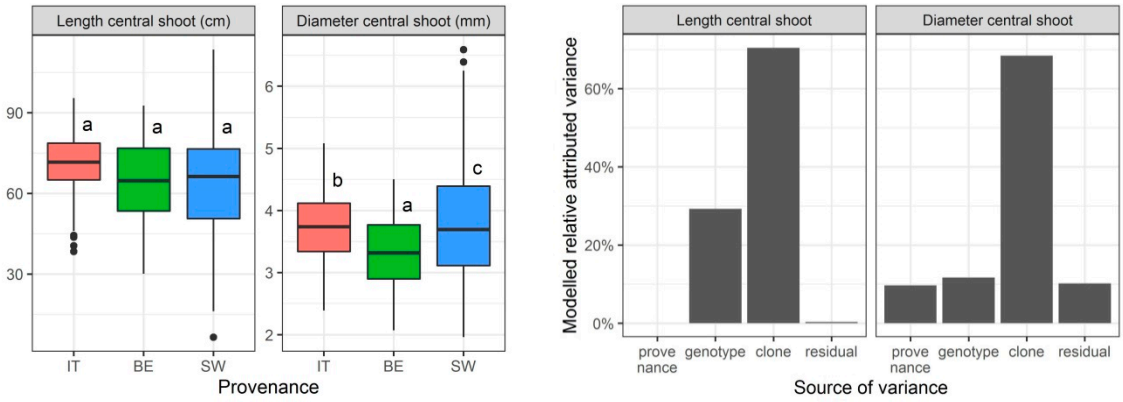


(**a**) (**b**)

Figure 4. Stomatal traits in the control plants. (**a**) Box plots for stomatal density and stomatal length. Letters indicate significant differences (Table S2); (**b**) Modelled distribution of the relative variance. Variance is explained by the factors provenances, genotypes within a provenance and the three nail varnish prints per genotype (image), for both the stomatal traits. IT: Italian provenance, BE: Belgian provenance, SW: Swedish provenance.

3.2. Height and Radial Growth during and after the Water Withholding Period

At the start of the drought treatment, there was no significant difference among the three provenances in terms of the length of the central shoot (Figure 5a, Table S3). However, the plants of both the Italian and the Swedish provenance had a significantly wider diameter than the plants of the Belgian provenance (p-values of 0.028 and <0.001 for P_I and P_S respectively, Table S3). In the analysis of the distribution of the relative variance, the largest part of the variation for both length and diameter growth was explained by the factor clones within a genotype, whereas the factors provenances and genotypes within a provenance explained only a lesser part (Figure 5b).



(**a**) (**b**)

Figure 5. Length and diameter of the central shoots at the start of the experiment (DOY 205). (**a**) Box plots of the measurements. Letters indicate significant differences (Table S3); (**b**) Modelled distribution of the relative variance. Variance is explained by the factors provenances, genotypes within a provenance and clones within a genotype. IT: Italian provenance, BE: Belgian provenance, SW: Swedish provenance.

The drought treated plants suffered from reduced growth, which is clearly visible when compared with the control plants (Figure 6). The length increment of the central shoot during the drought treatment was on average reduced with 17.6 cm (68%), 8.2 cm (52%) and 2.9 cm (37%) in the Italian, Belgian and Swedish provenances, respectively (Figure 6a). This reduction was significantly stronger in the Italian provenance than in the Belgian provenance (p-value < 0.001 for W:P_I in Table S4), whereas this reduction was significantly less in the Swedish provenance when compared with the Belgian provenance (p-value of 0.018 for W:P_S in Table S4). The reduction of the diameter increment of the central shoot during the drought treatment was on average 0.9 mm (97%), 0.5 mm (61%) and 0.5 mm (61%) for the Italian, Belgian and Swedish provenances respectively (Figure 6b) and this was significantly stronger in the Italian provenance compared with the Belgian provenance (p-value < 0.001 for W:P_I in Table S4), whereas no significant difference between the Belgian and the Swedish provenance (p-value = 0.724 for W:P_S in Table S4).

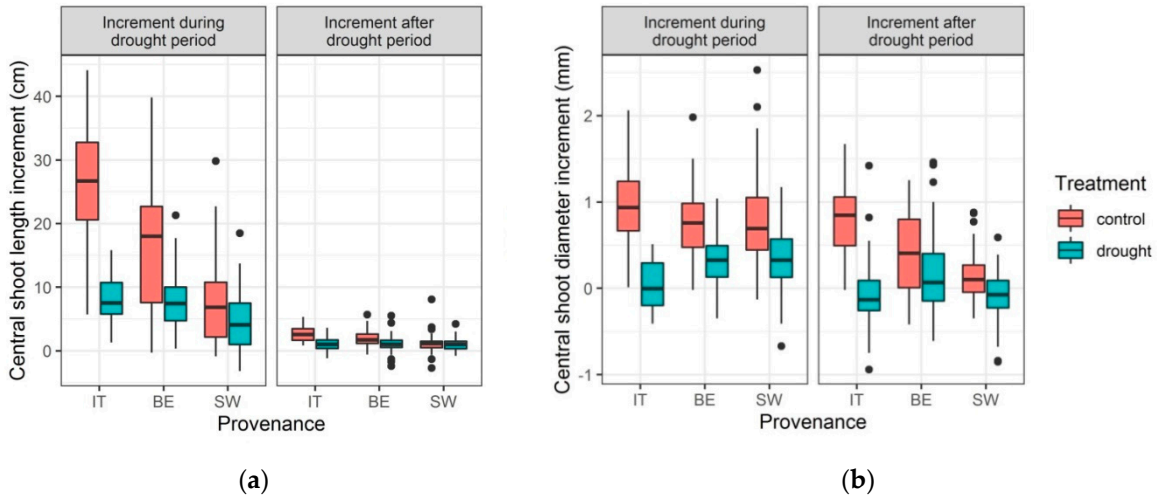


Figure 6. Growth increments for length and diameter of the central shoot during and after the water withholding treatment in 2018. (**a**) Box plots of the lengths of the central shoot; (**b**) Box plots of the diameters of the central shoot. IT: Italian provenance, BE: Belgian provenance, SW: Swedish provenance.

The length increment of the central shoot in autumn, after the re-watering, was limited in both the control and the treated group (Figure 6a). A reduction due to the water withholding treatment was observed of 1.6 cm, 0.8 cm and 0.1 cm on average for the Italian, Belgian and Swedish provenances, respectively. This reduction was significantly less in the Swedish provenance than in the Belgian provenance (p-value = 0.04 for W:P_S in Table S4), whereas the difference was not significantly different between the Belgian and Italian provenance (p-value = 0.08 for W:P_I in Table S4). Diameter increment after the re-watering was still affected by the drought treatment (p-value of 0.026 for W in Table S4). The reduction of the radial increment of the central shoot after the drought treatment was on average 0.9 mm, 0.2 mm and 0.2 mm for the Italian, Belgian and Swedish provenances, respectively (Figure 6b). These radial growth reductions were significantly different between the Belgian and the Italian provenance (p-value < 0.001 for W:P_I in Table S4), but no significant difference between the Belgian and the Swedish provenances (p-value = 0.659 for W:P_S in Table S4).

3.3. Timing of Leaf Wilting during the Water Withholding Period

Plants displayed stress symptoms during the water withholding period, with leaf wilting as the most obvious visible sign (Figure 7). Modelling the leaf wilting scores demonstrated that the Italian provenance was the first to show these symptoms, significantly differing from the Belgian provenance (p-value < 0.001 for P_I in Table S5, Figure 8), whereas the Swedish provenance did not significantly differ from the Belgian provenance (p-value = 0.380 for P_S in Table S5). In addition, the length of the central shoots played a significant role, depending on the relative weight loss of

the pots (p-value < 0.001 for W:S_1 in Table S5). At the end of the water withholding period 58.4% of the treated plants survived (Italian provenance: 7 plants belonging to 5 genotypes—21.9%, Belgian provenance: 45 plants belonging to 17 genotypes—68.2% and Swedish provenance: 38 plants belonging to 13 genotypes—67.9%).

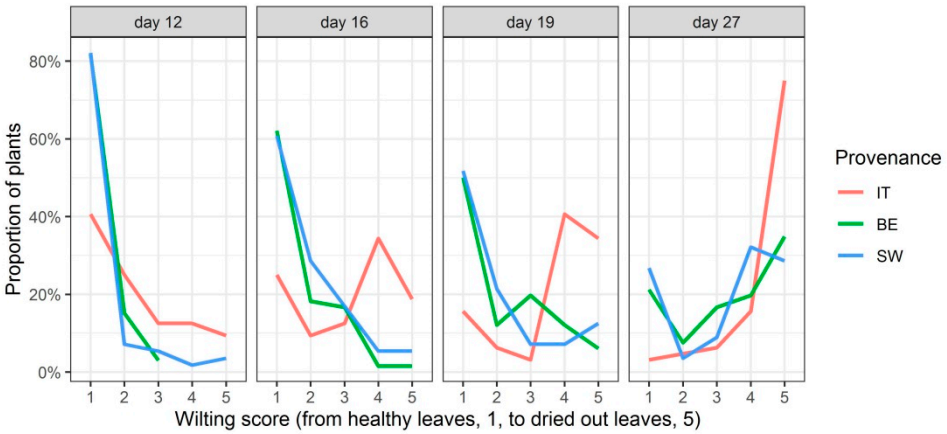


Figure 7. Leaf wilting scores during the water withholding treatment, observed on the 12th, the 16th, the 19th and the 27th day of the treatment. IT: Italian provenance, BE: Belgian provenance, SW: Swedish provenance.

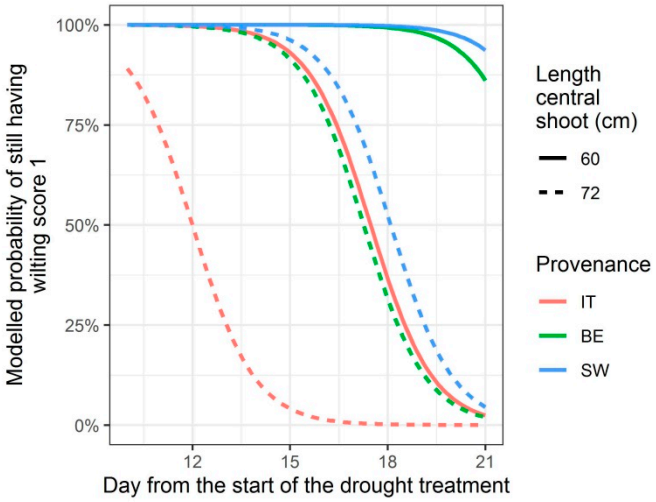


Figure 8. Modelled timing of leaf wilting for the drought treated plants, depending on the provenance and on the length of the central shoot at the start of the treatment. IT: Italian provenance, BE: Belgian provenance, SW: Swedish provenance.

3.4. Post-Drought Emergence of New Shoots

After the re-watering, new small shoots with new leaves emerged on the central shoots and/or on the base stems of 42% of the surviving plants (Figure 9). This phenomenon did not occur among the control plants. Modelling the absence/presence of these newly emerging shoots in the drought treated group revealed that only the length of the central shoot played a significant role in this process with plants with longer shoots having a higher chance for producing these new shoots (p-value = 0.005 for H_2 in Table S6). Provenance was not significant in the model. When considering the highest location on the plant where new shoots emerged, there was a tendency for bud burst in the next spring to occur at the same or a nearby location (Figure 9).

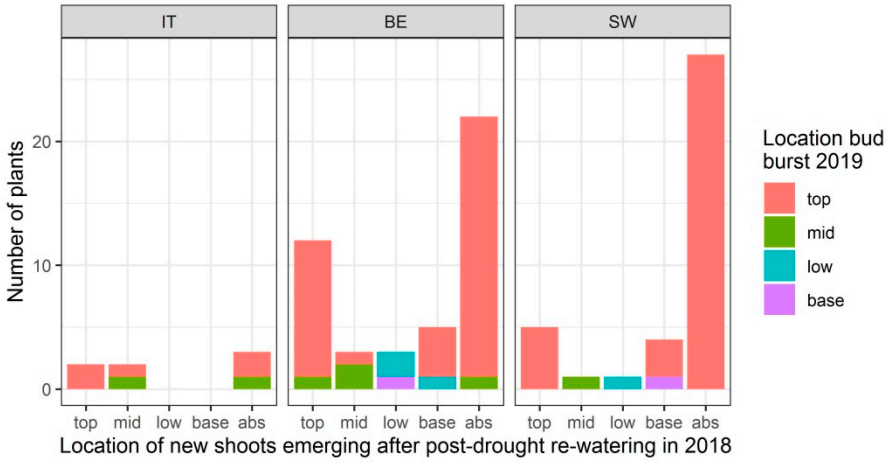


Figure 9. Presence/absence of new shoots with new leaves emerging after post-drought re-watering among the surviving plants of the treatment group, with location of the emerging new shoots on the plant, and relation with the location of the bud burst in the subsequent spring. Top: top part of the central shoot, mid: middle part of the central shoot, low: lower part of the central shoot, base: base stem, abs: absent. IT: Italian provenance, BE: Belgian provenance, SW: Swedish provenance.

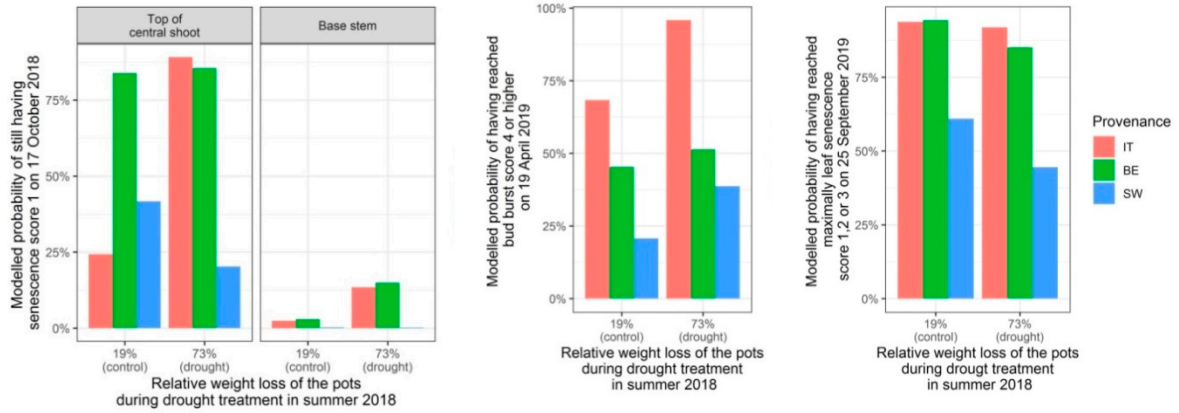
3.5. Post-Drought Phenophases

At the top of the central shoot, leaf senescence occurred earlier for both the Italian and the Swedish provenance in comparison with the Belgian provenance (p-values < 0.001 and = 0.028 for P_I and P_S respectively in Table S7). The water withholding treatment revealed a significant interaction term for the Italian provenance, indicating that the difference between non-treated and treated plants was bigger in the Italian provenance compared with this difference in the Belgian provenance (p-value of 0.037 for W:P_I in Table S7). In fact, the drought treated plants of the Italian provenance displayed a later leaf senescence compared to the control group of this provenance (Figure 10a), whereas for the Belgian provenance the difference in timing of leaf senescence between treated and control group was clearly less pronounced (Figure 10a). However, for the Swedish provenance, drought treated plants displayed a tendency to an earlier leaf senescence compared with the non-treated plants (p-value = 0.087 for W:P_S in Table S7, Figure 10a). At the base stem there was a significant effect of the drought treatment (p-value < 0.001 for W in Table S7) with the treated plants of the Italian and Belgian provenances having a later senescence compared with the control plants in these provenances (Figure 10a).

Bud burst in the apical bud, or the highest axillary bud when the apical bud had died off, in 2019 still displayed a significant difference between drought treated and control plants (p-value of 0.036 for W in Table S7, Figure 10b). Drought treated plants had an earlier bud burst (a higher probability of having reached a higher bud burst score in Figure 10b) in comparison with the control plants. The Swedish provenance was later to show bud burst compared with the Italian and Belgian provenance (p-value of 0.025 for P_S in Table S7). Bud burst at the base stem did not show the drought treatment signal anymore (no significance for W in Table S7).

The water withholding treatment was still significant in the leaf senescence scores in 2019 (W has a p-value < 0.001 in Table S7), with drought treated plants having an earlier senescence compared with the control (Figure 10c). The different provenances did not differ in their response on the treatment (no significant interaction terms of P with W in Table S7). The Swedish provenance displayed an earlier leaf senescence in comparison with the Italian and Belgian provenance (p-value < 0.001 for P_S in Table S7).

Bud burst in 2020 was not affected anymore by the treatment. There was no differentiation anymore between the three provenances.



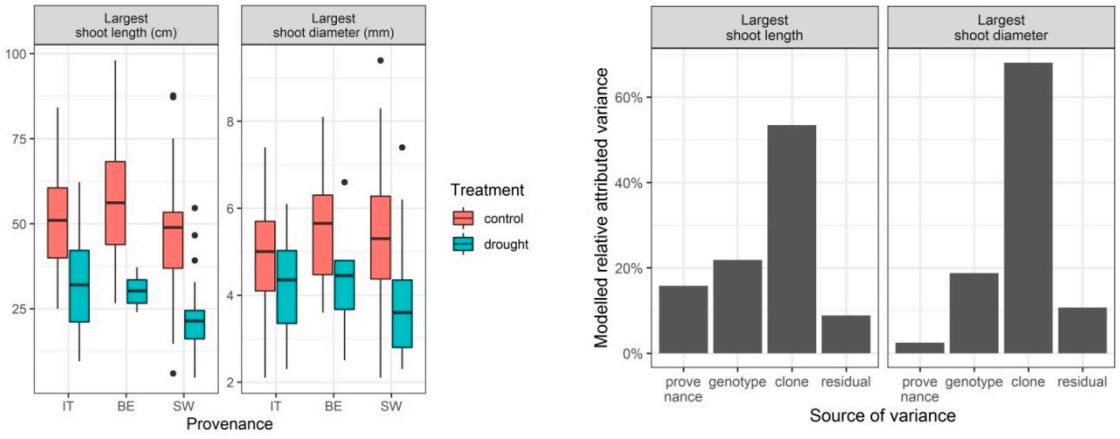
(**a**) (**b**) (**c**)

Figure 10. Modelled scoring of the phenophases, depending on the provenance and on the relative weight loss of the pots. (**a**) Post-drought leaf senescence in 2018 scored on two locations of the plant: the top of the central shoot and the base stem; (**b**) Bud burst in 2019 scored on the top of the central shoot; (**c**) Leaf senescence in 2019. IT: Italian provenance, BE: Belgian provenance, SW: Swedish provenance.

3.6. After Effects on Shoot Length, Radial Growth and Plant Architecture

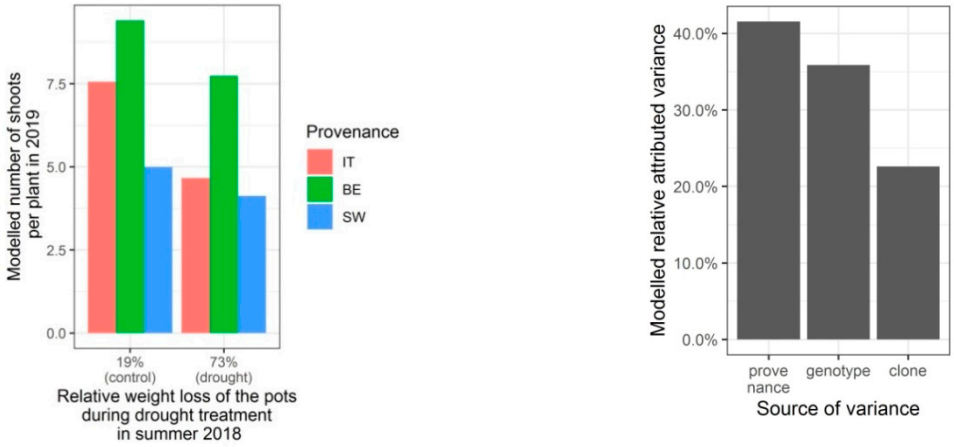
In 2019, the year succeeding the drought treatment, plant growth and plant architecture were examined upon pruning back all plants to the base stem immediately after the bud burst. The length and the diameter of the newly emerged largest shoot on each plant were still significantly affected by the drought treatment (W has a p-value < 0.001 for length and a p-value of 0.001 for diameter in Table S8, Figure 11a) but, without differentiation between the three provenances. The distribution of the modelled variance indicated that the factors provenance and genotypes within a provenance (both genetically controlled) together explained less than the factor clones within a genotype, pointing to a relative low genetic control (Figure 11b).

The Swedish provenance had significantly less shoots per plant that emerged on the base stem after the pruning than the Belgian provenance (p-value < 0.001 for P_S in Table S9, Figure 11c). The drought treatment still affected the number of shoots per plant (p-value of 0.010 for W in Table S9), with less shoots for the treated plants (Figure 11c). The distribution of the modelled variance for the number of shoots per plant showed that the factor provenances and genotypes within a provenance explained more than the factor clones within a genotype (no residual variance for generalized mixed models), pointing to a relative strong genetic control (Figure 11d).



(**a**) (**b**)

Figure 11. *Cont.*



(**c**) (**d**)

Figure 11. Size of the largest shoot and plant architecture in 2019. (**a**) Length and diameter of the largest shoot per plant at the end of 2019; (**b**) Distribution of modelled relative variance for the size measurements on the largest shoot per plant, with the factors provenances, genotypes within a provenance and clones within a genotype; (**c**) Modelled number of shoots per plant, depending on the provenance and on the relative weight loss of the pots during the treatment in 2018; (**d**) Distribution of modelled relative variance between the provenances, between the genotypes within a provenance, between the clones within a genotype and residual variance for number of shoots per plant. IT: Italian provenance, BE: Belgian provenance, SW: Swedish provenance.

4. Discussion

4.1. Quicker Drought Stress Symptoms in a Southern European Provenance

As the climate at the home-site of the Italian provenance is hotter than the Belgian (local) and Swedish provenances [44], we hypothesized a better thermo-tolerance and a likely concurrent better drought tolerance in the Italian provenance in our common garden experiment. In a former study, adaptive molecular markers (Single Nucleotide Polymorphisms) were associated with mainly temperature as an environmental factor in a comparison of the three provenances, indicating adaptation to local climate [42]. A better drought tolerance was suggested for Italian populations of *Q. robur* L. and *Q. petraea* compared with German and South African populations based on tree ring-climate correlations [53]. The Italian populations displayed the least radial growth reduction in the two years following the drought event [53]. Therefore, the effects that the Italian provenance displayed in response to drought stress in our experiment, was opposite to our expectation. The Italian provenance grew faster than the other provenances and produced larger leaves with a higher stomatal density but with no difference in stomatal size. The variance analysis showed that leaf size and stomatal density are relatively stronger genetically controlled than shoot size and stomatal size respectively. The larger leaf sizes in the Italian provenance seem opposite to the general idea that leaf area reduction is an adaptation to drought [54,55].

An important mechanism controlling the water balance and leaf gas exchange in trees is stomatal opening and closure [56]. Plants with large and few stomata tend to have higher water-use efficiency [57], but also reduced rates of photosynthesis [58]. The Italian provenance in our common garden was expected to display smaller sized leaves with a lower stomatal density as an expression of adaptation to a hotter home-site climate, which was not the case. Stronger growth for a more southern provenance in a common garden also seems to contradict the general trade-off between drought tolerance and growth which is described in interspecies comparisons [59]. Possibly, the translocation of the Italian provenance to an environment with sufficient water supply and a temperate Atlantic climate (potted plants grown outdoors under a regular irrigation regime prior to the experiment) may have caused a

phenotypic plastic response to the favorable growth environment with a stronger growth and larger leaves when compared with the local provenance. In the drought treated group, this plastic response with a larger growth and a likely higher transpiration through larger leaves with higher stomatal density may have led to the quicker and more severe drought stress damage in this provenance. This plastic "over-reaction" to favorable growth conditions in a provenance putatively adapted to more harsh growing conditions can be related to the findings of Hajek, et al. [60]. In this study wood anatomical traits in 10 different provenances of *F. sylvatica* grown in a common garden in Northern Europe were compared. The Southern European provenance of Spain displayed the largest vessel sizes, which was contrary to expectations, as large vessel sizes are generally correlated to a stronger sensitivity to vessel embolism under drought stress, whereas a better drought tolerance of this Southern European provenance, adapted to a harsher climate at the home-site, was anticipated.

4.2. Compensation Mechanisms and Other after Effects

A remarkable after effect of the summer drought was the appearance of new shoots upon re-watering in several drought treated plants, at different locations on the central shoot and base stem, but without differentiation among the three provenances. In normal conditions, axillary bud outgrowth is associated with apical dominance, which is well-known to be controlled by the phytohormone auxin, whereas cytokinins have been identified as the phytohormones promoting axillary bud outgrowth [61]. In the model plant *Arabidopsis thaliana* (L.) Heynh., a high elevation of active cytokinins was found during post-drought stress recovery [62]. In this sense, we can hypothesize that an up-regulation of cytokinins may have caused the reshooting from axillary buds upon re-watering in our experiment. It is assumed that enhanced plant growth during post-drought re-watering is a compensation for the loss of net primary production during the water deficit and this has been observed in herbaceous plants such as perennial grasses [46] and in woody species such as *Q. robur* L. and *Q. petraea* [36,63]. In perennial grasses, the magnitude of the post-drought increase in relative growth rate was correlated with the severity of the drought [46]. The higher chance of forming an extra growth shoot in *Q. petraea* upon re-watering after a summer drought period can be related to our finding [63]. However, the recovery reshooting in our *F. alnus* plants was axillary whereas the extra shoot formation in *Q. petraea* was apical, leading to a higher mean height of drought treated seedlings in the recovery phase [63], and may be attributed to *Q. petraea* being a dominant tree species whereas *F. alnus* is an understory shrub.

In the top part of the plant, the Italian provenance displayed a clear delay of autumnal leaf senescence in response to the drought whereas at the level of the base stem, this phenomenon was observed in both the Italian and the Belgian provenance. Amongst other factors, drought is known to induce leaf senescence. Drought-induced senescence reduces nutrient losses from temperate deciduous trees under severe drought [64]. Contrastingly, a delayed senescence upon post-drought re-watering was observed in saplings of *Q. petraea* [39] and *F. sylvatica* [40], and was suggested to create recovery time before entering autumnal leaf senescence [39]. The delayed senescence most strongly expressed in the Italian provenance can be attributed to the likely relatively stronger stress endured by these plants. Similarly, the increase in relative growth rate in the post-drought recovery phase in perennial grass could be correlated to the severity of the experienced drought stress [46]. The Swedish provenance entered autumnal senescence earlier than the Belgian and the Italian provenance in the two years of observation, expressing adaptation to a shorter growing season. As the natural autumnal leaf senescence started earlier in this provenance, the senescence delaying effect of the post-drought re-watering may not have had the chance to develop.

Bud burst at the top of the plants was advanced in the treatment group, for all three provenances. An earlier start of the growing season can also be interpreted as a post-drought recovery mechanism intended to promote growth as a compensation for the lost growing time. Still a reduced growth (shoot length and diameter) was detected in the growing year following the drought treatment. Drought stress is well-known to hamper resource storage needed for subsequent growth [65]. This reduced growth was also likely attributing to the observed advanced leaf senescence in this year. The impact of

the water withholding period on the phenophases disappears in the bud burst of the second year after the treatment.

A last interesting observation concerned the plant architecture. Plants resprouted upon pruning after the bud burst in the year following the treatment, with significantly less numbers of shoots per plant for the treated group of plants. No differentiation was observed among the three provenances, while the variance analysis indicated a relative strong genetic control for this trait. In *Q. robur* and *Q. petraea* saplings that were pruned four years after a summer drought treatment, the number of reshoots per plant was not influenced anymore by the drought treatment, but the number of side shoots on the reshoots was significantly lower in the drought treated group [66]. The phytohormone strigolactone was hypothesized to be involved in this longer term response to drought, which was interpreted as a promotion of height growth over side branching. Strigolactones are a recently described class of phytohormones regulating shoot branching [67,68]. In *A. thaliana*, strigolactones were shown to cross-talk with the plant hormone abscisic acid which is involved in signaling drought responses [69]. An influence of this phytohormone can also be hypothesized in the post-drought resprouting after pruning of the *F. alnus* plants in our experiment. With a given amount of recourses per plant, the allocation of resources to a lesser amount of resprouts allows the plant to attain a higher height compared with the formation of a higher number of resprouts, which can provide the plant with an advantage in a competitive growth environment.

5. Conclusions

As a climate adaptation strategy, more southerly provenances of woody species are being suggested to be translocated to more northerly sites. As provenances at the southern parts of the natural range of tree species have generally maintained relatively large levels of genetic diversity and tolerance to warmer and drier environment, they are suggested as suitable gene pools for assisted migration towards the north [5]. Instead, results from common garden trials for several shrub species in Northern Europe indicate substantial genetic variation and evolutionary potential in local populations, questioning the need for assisted migration for wide-spread and generally occurring woody species [70]. Our results add an argument to this on-going debate. When translocating provenances of woody plants, both genetic adaptation to the home-site and phenotypic plastic responses to the new growth environment may interact, which may not always lead to the expected growth patterns and tolerances to biotic and abiotic stresses. Our results underline the value of carefully reflecting on assisted migration, and not automatically excluding local planting stock.

Supplementary Materials: The following are available online at http://www.mdpi.com/1999-4907/11/11/1186/s1, Figure S1: Relative weight loss of the pots for each provenance and according to the treatment of the plants. IT: Italian provenance, BE: Belgian provenance, SW: Swedish provenance. Table S1: Model statistics for leaf lamina length and lamina widest width in the control group. P is the provenance, with the Belgian provenance as the standard level to which the Italian (P_I) and Swedish (P_S) provenance are compared with. H_2 is the length of the central shoot at the end of the drought treatment. Table S2: Model statistics for stomatal density and stomatal length. P is the provenance, with the Belgian provenance as the standard level to which the Italian (P_I) and Swedish (P_S) provenance are compared with. H_2 is the length of the central shoot at the end of the treatment. L is the length of the leaf lamina. Table S3: Model statistics for the length and diameter measurements of the central shoot at the start of the treatment. P is the provenance, with the Belgian provenance as the standard level to which the Italian (P_I) and Swedish (P_S) provenance are compared with. Table S4: Model statistics for the growth increments of the length and the diameter of the central shoot during and after the water withholding period in 2018. P is the provenance, with the Belgian provenance as the standard level to which the Italian (P_I) and Swedish (P_S) provenance are compared with. W is the weight loss of the pots during the water withholding treatment. H_1 and H_2 are the lengths of the central shoots at the start and at the end of the treatment respectively. Table S5: Model statistics for the timing of the wilting of the leaves during the drought treatment of the plants. P is the provenance, with the Belgian provenance as the standard level to which the Italian (P_I) and Swedish (P_S) provenance are compared with. W is the weight loss of the pots during the water withholding treatment. H_1 is the length of the central shoot at the start of the treatment. Table S6: Model statistics for the binary response variable presence/absence of new emerging shoots after post-drought re-watering. P is the provenance, with the Belgian provenance as the standard level to which the Italian (P_I) and Swedish (P_S) provenance are compared with. H_2 is the length of the central shoot at the end of the treatment. W is the weight loss of the pots during the water withholding treatment. Table S7: Model statistics for the post-drought phenophases leaf senescence and

bud burst. P is the provenance, with the Belgian provenance as the standard level to which the Italian (P_I) and Swedish (P_S) provenance are compared with. H_3 and H_4 are the length of the central shoot at the end of 2018 and the length of the largest shoot at the end of 2019 respectively. W is the weight loss of the pots during the water withholding treatment. Table S8: Model statistics for the largest shoot size measurements at the end of 2019. P is the provenance, with the Belgian provenance as the standard level to which the Italian (P_I) and Swedish (P_S) provenance are compared with. W is the weight loss of the pots during the water withholding treatment. Table S9: Model statistics for the number of reshoots per plant at the end of 2019. P is the provenance, with the Belgian provenance as the standard level to which the Italian (P_I) and Swedish (P_S) provenance are compared with. W is the weight loss of the pots during the water withholding treatment. H_4 is the length of the largest shoot at the end of 2019.

Author Contributions: K.V.M., J.V.d.B., M.C., L.D.L. and K.S. conceptualized this study and worked out the methodology. K.V.M., J.V.d.B. and L.D.L. supervised the whole study, while S.M., together with L.D.C. and N.V.d.S., organized the cutting, the pruning and the plant growth in the nursery and in the greenhouse. L.D.C., N.V.d.S., S.M., A.L. and K.V.M. performed observations and measurements. L.D.C., A.L., N.V.d.S., J.V.d.B., L.D.L. and K.V.M. conducted the statistical analyses. K.V.M., J.V.d.B., M.C., L.D.L. and K.S. prepared the manuscript. All authors have read and agreed to the published version of the manuscript.

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